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RESEARCH PAPER

Antisense suppression of a β-galactosidase gene (*TBG6*) in tomato increases fruit cracking

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Abstract

Antisense suppression of a tomato β-galactosidase gene (*TBG6*) was used to study its role in fruit development, cell wall-modification, and fruit firmness. *TBG6* mRNA is highly abundant during the early stages of fruit development, but the levels decline sharply after the breaker stage with the start of the respiratory climacteric and a concomitant increase in ethylene production. Two antisense lines were obtained with significantly reduced levels of *TBG6* mRNA at all stages of fruit development. At 30 d after pollination (dap), *TBG6* mRNA levels were reduced by up to 98% and 88% in lines 6-2 and 6-10, respectively. Morphological phenotypes observed in the antisense lines included increased fruit cracking, reduced locular space, and a doubling in the thickness of the fruit cuticle. Two biochemical changes in antisense lines, compared with wild-type lines, were a reduction of exo-galactanase activity at the breaker +3 d stage and a reduction in the cell wall galactosyl content at the 20 dap stage. In addition, transgenic lines exhibited a 35–39% reduction in fruit firmness at the 20 dap stage, but their texture was equivalent to the wild type at 30 dap and beyond. Although the exact function of the TBG6 product is still unknown, these results implicate an important role for this enzyme in early fruit growth and development in tomato.

Key words: Antisense suppression, fruit cracking, tomato.

Introduction

Fruit growth and development occur as a result of a myriad of biochemical and physiological events. The texture of the tomato (*Lycopersicon esculentum* Mill.) fruit, for example, is controlled by cell turgor pressure and changes in cell wall structure and composition. Several cell wall-modifying enzymes active during ripening have been identified. Some cell wall-degrading enzymes include polygalacturonase (PG), pectinesterase (PE), expansin, and β-galactosidase (Brummell and Harpster, 2001). Decreasing levels of PG and PE by transgenic antisense technology significantly altered traits such as cracking and viscosity, but had little effect on fruit firmness (Schuch *et al*., 1991). On the other hand, suppression of the ripening-related expansin Exp1 and tomato β-galactosidase 4 (TBG4) resulted in significantly firmer fruit (Brummell *et al*., 1999; Smith *et al*., 2002).

β-Galactosidases are a large family of glycosyl hydrolases that reduce the levels of cell wall galactosyl residues in ripening tomato fruit (Gross and Sams, 1984; Smith *et al*., 1998; Carey *et al*., 2001). Genes from a family of at least seven tomato β-galactosidases (TBG) show unique patterns of expression during fruit development (Smith and Gross, 2000). Antisense technology has been used to study these genes and promises to be a useful tool in attempts to produce fruit with attenuated softening. Down-regulation of *TBG1* and *TBG3* did not result in significant modifications in fruit firmness: however, reduced levels of *TBG3* did result in lower levels of enzyme activity, altered cell wall composition, and increased serum viscosity of processed paste (Carey *et al*., 2001; de Silva and Verhoeyen, 1998). Antisense down-regulation of *TBG4* resulted in fruit that were up to 40% firmer than controls, thus confirming a role for this gene's product in determining fruit firmness (Smith *et al*., 2002). Further antisense studies of this nature are needed to elucidate the function(s) of the remaining members of the TBG family.

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Abbreviations: Br+3, Breaker stage plus 3 d; dap, days after pollination; MG, mature green stage; TBG, tomato b-galactosidase.

Among the TBG genes, *TBG6* exhibits the highest expression levels, but only during the green stages of tomato fruit development, prior to visible signs of ripening (Smith and Gross, 2000). At the start of the breaker stage, the levels of *TBG6* mRNA decline dramatically and little or no mRNA (depending on the cultivar) is detected during ripening (from breaker to the red ripe stage). The climacteric rise of ethylene in ripening tomato fruit may play a role in the developmental down-regulation of *TBG6*; wild-type fruit treated with ethylene at 25 and 33 dap show a dramatic decrease in *TBG6* mRNA accumulation (Moctezuma *et al*., 2003). cDNA nucleotide and deduced amino acid sequence data predict the TBG6 product to be 91 kDa and secreted from the cell (Smith and Gross, 2000). Because of its unique temporal expression pattern and likely presence in the wall during fruit development, it is suspected that TBG6 is involved in early fruit growth (possibly wall expansion in immature fruit) and/or modifying fruit texture. To determine TBG6's function in fruit growth and development, morphological and anatomical changes, cell wall modification, and fruit firmness were studied in antisense transgenic plants with suppressed *TBG6* mRNA level.

Materials and methods

Plant material

Tomato plants (*Lycopersicon esculentum* Mill. cv. 'UC82B') were grown in a greenhouse using standard cultural practices. Flowers were pollinated at anthesis using a vibrating device, and fruit were harvested at 10, 20 and 30 d after pollination (dap), as well as 3 d after the breaker stage (Br+3). The breaker stage was determined by incipient red coloration at the blossom end of the fruit. For molecular and biochemical analysis, five fruit from at least three plants of each line were collected and processed together by mixing peel and pericarp tissues and removing samples for different analysis.

Transformation vector and transgenic plant selection

The transformation vector was constructed by modifying the binary vector PBI121 (Clontech, Palo Alto, CA). The β-glucuronidase coding region was first removed by digesting with *Bam*HI and *Sac*I, then a 749 bp fragment (nt 254–1003) from the 5′ end of the *TBG6* cDNA (accession number AF154424) was inserted in the antisense orientation with respect to the 35S CaMV promoter. Seven-day-old cotyledons were transformed using *Agrobacterium tumefaciens* (strain LBA4404) and plantlets were regenerated as in Deikman and Fischer (1988). Transformation was confirmed by DNA gel-blot analysis, using genomic DNA isolated from T_0 plants. Seeds from six T_1 generation plants were grown on kanamycin-containing media to select for antibiotic-resistant plants with the T-DNA insertion. After preliminary RNA gel-blot analysis of *TBG6* mRNA suppression, two antisense lines (6-2 and 6-10) were selected for further study. Control plants were the parental wild type and azygous, kanamycin-sensitive T_1 plants of lines 6-2 and 6-10, which were rescued from kanamycin-containing media.

RNA gel-blot analysis

Control and transgenic fruit were harvested at 10, 20, and 30 dap and Br+3, and 4 g of pericarp and peel tissue (combined) were chopped and immediately frozen in liquid nitrogen. Total RNA extraction and gel-blot analysis were as described in Smith and Gross (2000). Briefly, 20 μg of total RNA per sample were separated in a formaldehyde-agarose (1.5%) gel, transferred to a Hybond-N^+ nylon membrane (Amersham, UK), and fixed by incubating for 2 h at 80 °C. Membranes were washed in 0.3 M Na-acetate (pH 5.2), methylene blue and 0.2× SSC (0.5% SDS) as described in Smith *et al*. (2002). *TBG6*-specific probes were synthesized using DNA fragments derived from PCR-amplification of the cDNA nucleotides 254–1003 (to hybridize to antisense and sense mRNA) and nucleotides 2551–3023 (to hybridize to sense mRNA 3′UTR only) using a Random Primed DNA Labeling Kit (Roche, Indianapolis, IN) with [^{32}P]dATP (3000 Ci mmol^{-1}) as the label. Probes for *TBG4* and *TBG5* were synthesized as described in Smith *et al*. (2002). Biomax MS X-ray film (Kodak, Rochester, NY) was used to expose the blots in an intensifying screen for 36 h at –80 °C. Finally, blots were stripped and hybridized using an apple 18S rDNA fragment to allow normalization for differences in loading of total RNA in each lane. Autoradiographs were quantified using a Fluor-S MultiImager scanner and analysed with Quantity One software (Bio-Rad, Hercules, CA). To corroborate the results, RNA gel blot experiments were repeated twice.

Anatomical sections, cracking and cuticle measurements

Peel/pericarp tissue samples were collected from five fruit of wild type and line 6-2 at the mature green and red stages of development. The tissue samples were fixed in 50% formalin-acetic acid-alcohol (FAA) under a vacuum for 16 h. Next, tissue samples were dehydrated in an increasing concentration series of ethanol:water, infiltrated with CitriSolv (Fisher, Pittsburgh, PA), embedded in paraffin, and sectioned at 12 μm on a rotary microtome. Sections were mounted on glass slides and stained with aqueous Safranin O (1%) and Fast Green (0.1% in 95% ethanol), as described by Ruzin (1999). Safranin O stains cutin, a major component of the cuticle. The thickness of the cuticle of each sample was measured using a calibrated light microscope at 100× magnification. Three tissue samples (blocks) from each fruit were processed. Five to ten measurements were made from each section for a total of 64 and 80 measurements for wild type and line 6-2, respectively.

Per cent fruit cracking was measured on 6-10 plants per line. An average of 15 fruit per plant (more than 100 total fruit per line), at all stages of development, were visually examined for cracks or scars on the surface of the pericarp. Typically one to three cracks and/or scars were found per fruit.

Enzyme assays and cell wall analysis

Cell wall-associated enzymes were extracted from tomato fruit peel and outer pericarp tissue according to the technique described in Carey *et al*. (1995) and Kim *et al*. (1991). Briefly, 20 g of fruit pericarp tissue were homogenized in a blender with 30 ml of water, NaCl was added to 1 M, and the pH adjusted to 6.0. After filtration and centrifugation, $(NH_4)_2SO_4$ was added to 80% saturation. A suspension of precipitated proteins was dialysed against water for 4, 16 and 2 h at 4 °C. For estimating β-galactosidase activity, *p*-nitrophenol-β-D-galactopyranoside (PNP-gal) was used as a substrate (Pressey, 1983), and one unit of activity was defined as the amount of enzyme that liberated 1 μmol PNP min^{-1} at 37 °C. For exo-galactanase activity, lupin galactan (Megazyme, Wicklow, Ireland) was used as a substrate, following the method described by Carey *et al*. (1995) and Smith *et al*. (2002). Assays were run under conditions in which product formation was linear with time and proportional to the amount of enzyme in the assay.

Cell wall preparations were obtained from peel and outer fruit pericarp tissue using the methods described by Gross (1983). Ten grams of fruit pericarp tissue from wild-type and transgenic lines at different stages of development were chopped, frozen immediately

in liquid nitrogen, and pulverized. The glycosyl composition of the acetone-insoluble cell wall material was established by hydrolysis with 2 N TFA followed by gas chromatography–mass spectrometry–selected ion monitoring of the alditol acetate derivatives of the monosaccharides released by hydrolysis (Gross and Acosta, 1991). Each enzyme assay and cell wall analysis was performed twice.

Texture measurements

Firmness measurements were taken from 12–20 whole fruit from each line, harvested at 20 and 30 dap. Each fruit was tested one to three times by indentation with a 4 mm diameter steel cylinder probe centred on the middle of a locule, as described in Smith *et al*. (2002). Texture measurements were determined using an instrument (TA-XT2i, Stable Microsystems Texture Analyser, Surrey, UK) loading at 1 mm s^{-1} to an indentation of 3 mm in depth. Firmness was defined as the maximum force required to deform the pericarp tissue 3 mm. Statistical analysis of maximum force measurements was performed using SAS software (SAS Institute, Cary, NC), and the statistical significance of the results was determined using the Tukey–Kramer multiple comparison test.

Results

Antisense suppression of TBG6

Tomato plants were transformed with an antisense-oriented *TBG6* cDNA fragment and a gene for kanamycin resistance. After regeneration, several T_0 lines were chosen and grown to maturity. From these lines, T_1 seed were collected and plated onto kanamycin-containing growth medium. Lines having a 3:1 resistant:sensitive ratio, meaning they had a single locus insertion of the T-DNA, were chosen for further study. Two lines (6-2 and 6-10) were selected and DNA gel-blot analysis was performed in these lines to confirm the T-DNA copy number and integrity (data not shown). Both the wild-type line used for transformation and an azygous line derived by rescuing T_1 kanamycin-sensitive plants from lines 6-2 and 6-10 served as controls.

RNA gel-blot analysis was carried out using the 3′UTR as a probe to detect *TBG6*-specific sense mRNA only. In wild-type and azygous fruit, *TBG6* mRNA levels were high in the early stages of development (10, 20 and 30 dap), but then dropped dramatically after the breaker stage (Fig. 1). Fruit from transgenic line 6-3 had levels of *TBG6* mRNA similar to wild type, whereas lines 6-2 and 6-10 had significantly reduced levels of *TBG6* mRNA. In antisense line 6-2, mRNA levels of *TBG6* are greatly down-regulated at all stages of development; up to 98% suppression at 30 dap. Line 6-10 showed moderate suppression of *TBG6* mRNA at the early stages of development: approximately 93% and 88% suppression at 20 and 30 dap, respectively (Fig. 1). An additional RNA gel-blot was hybridized using a probe that hybridizes to both sense and antisense *TGB6* mRNAs. All antisense lines had abundant levels of antisense transcript at all stages of fruit development (data not shown).

Although unlikely, since a segment from the ORF of the *TBG6* cDNA was used to construct the antisense cassette,

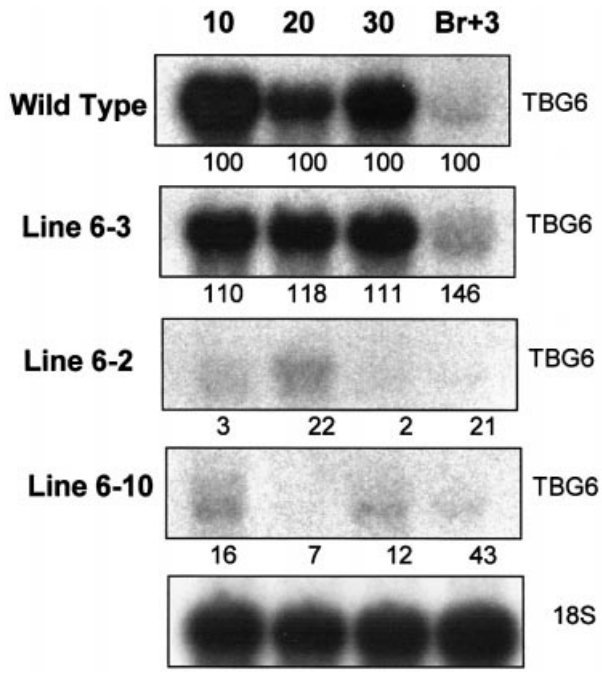

Fig. 1. RNA gel-blot analysis of *TBG6* mRNA abundance in the wild type and in transgenic lines 6-3, 6-2 and 6-10 at 10, 20 and 30 d after pollination (dap) and breaker plus 3 d (Br+3) stage. Twenty micrograms of total RNA extracted from fruit at the above stages was loaded on each lane. Blots were hybridized using the probes indicated on the right. Wild-type *TBG6* mRNA levels were arbitrarily set at 100, and the numbers under each lane represent the per cent band density compared to the wild type. An 18S ribosomal gene clone from apple was used as a loading control for scanning densitometry and one example is shown.

it was possible that other β-galactosidase gene family mRNAs might be suppressed as well, especially *TBG4* and *TBG5* (which share significant sequence identity) are expressed before ripening, and have exo-galactanase activity. The line with the greatest suppression of *TBG6*, line 6-2, was therefore tested for *TBG4* and *TBG5* mRNA abundance. It was found that *TBG4* mRNA levels were similar to wild-type levels before ripening (Fig. 2). However, *TBG4* mRNA levels in line 6-2 were 43% lower than wild type at the Br+3 stage. Levels of *TBG5* mRNA in line 6-2 were similar to wild-type levels except for a slight increase at 20 dap.

Morphological and anatomical changes

Fruit from transgenic line 6-3 (with normal levels of *TBG6* mRNA) had no altered physiological, biochemical or morphological phenotypes. However, several morphological and anatomical changes were observed in fruit of the transgenic lines with suppressed *TBG6* mRNA. Transgenic lines 6-2 and 6-10 exhibited cracks in the epidermis of the fruit at early stages of development. These cracks eventually healed and formed ‘zipper-like’ scars (Fig. 3A). Most scars were oriented vertically, however, they also appeared with a horizontal or diagonal orienta-

tion. Anatomical sections revealed that the cracks and scars were several cell layers deep, of possible internal origin, and that they may involve changes in the cell wall (lignification, cutinization, etc.) (Fig. 3B). The number of fruit with one or several scars, as a percentage of the entire fruit per plant, increased with increased *TBG6* suppression: 40% of the fruit of line 6-2 exhibited cracking, whereas approximately 14.5% of fruit in line 6-10 did so (Fig. 3C). In the wild-type and azygous lines only 1.8% or 1% of fruit, respectively, exhibited surface cracks. In addition, in transgenic line 6-3 (which has normal levels of *TBG6* mRNA) only 3.3% of fruit had cracks.

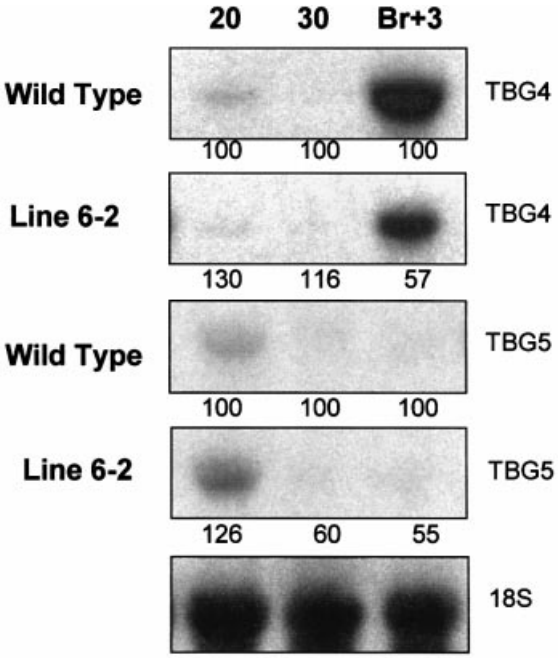

Fig. 2. RNA gel-blot analysis of *TBG4* and *TBG5* mRNA levels in the wild type and in the antisense line 6-2, which exhibits the greatest *TBG6* suppression. Twenty micrograms of total RNA extracted from fruit at 20 and 30 dap and breaker plus 3 d (Br+3) was loaded in each lane. Blots were hybridized using the probes indicated on the right. Wild-type *TBG4* and *TBG5* mRNA levels were arbitrarily set at 100, and the numbers under each lane are the per cent band density compared to the wild type. As a loading control for scanning densitometry, an 18S ribosomal gene clone from apple was used and one example is shown.

The internal fruit anatomy of transgenic lines 6-2 and 6-10 was characterized by a lack of open locular space (Fig. 4). In cross-section, fruit of lines 6-2 and 6-10 consistently had a smaller open locular space than the wild type or azygous lines, possibly due to a thicker pericarp and larger columella and locules. Despite the increased volume of internal tissues in line 6-2, the average number of seeds per fruit was approximately 80% lower than the wild type (data not shown).

One of the most dramatic phenotypes observed in the transgenic lines was the glossiness of the fruit surface. This phenotype was first observed by the inability to write on green fruit from antisense lines 6-2 and 6-10 with a felt-tip permanent marker. Whereas ink flowed smoothly on the surface of control and line 6-3 fruit, the ink formed small droplets on the surface of fruit from lines 6-2 and 6-10. Upon further anatomical analysis, a doubling in the thickness of the cuticle at the mature green stage was found (Fig. 5). The cuticle of the parental wild type and azygous lines measured approximately 4.96 µm, whereas the cuticle of line 6-2 measured 10.06 µm. As a probable consequence of the thicker cuticle, transgenic fruit at the mature green stage appeared glossier than the wild type. It is interesting to note that this difference in cuticle thickness was seen only during the green stages of development: the cuticle at the red stage of development was not statistically different in control and transgenic fruit. The cuticle measured 7.7 µm (SE=1.07) in the wild type, and 8.3 µm (SE=1.29) in line 6-2, during the red ripe stage of development.

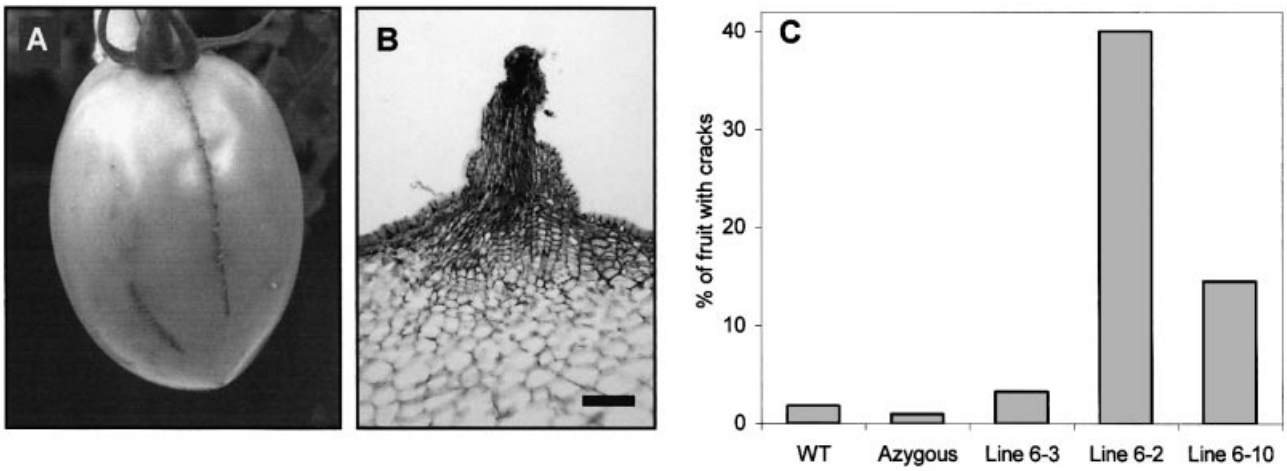

Fig. 3. Morphological and histological analysis of transgenic line 6-2. (A) Fruit from line 6-2 showing the cracking phenotype. (B) Paraffin section of a crack in the fruit pericarp surface (bar=100 µm). (C) Per cent of fruit exhibiting cracking in azygous, wild-type and transgenic lines.

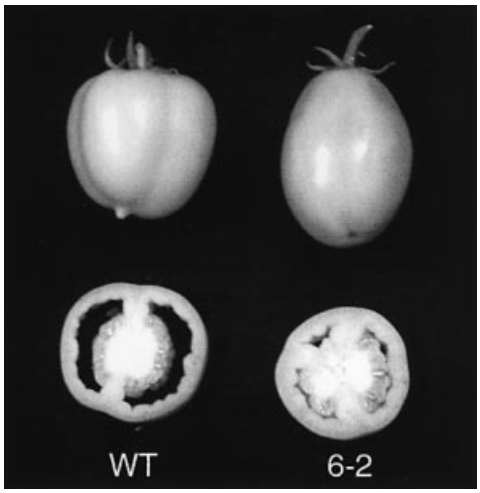

Fig. 4. External and internal tomato fruit morphology of the wild type and line 6-2. Notice the more elongated fruit shape and the decreased open locular space in the transgenic line.

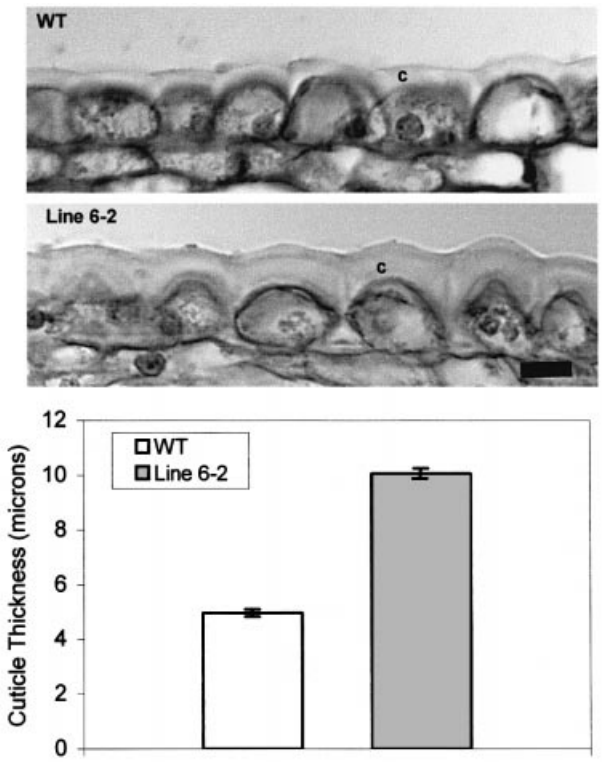

Fig. 5. Paraffin sections of the epidermis of mature green stage fruit from the wild type (top) and the transgenic line 6-2 (middle) plants. The cuticle is marked with the letter c (bar=10 μm). The graph (bottom) shows that the cuticle is twice as thick in line 6-2 (10.06 μm) than in the wild type (4.96 μm) (±SE).

Physiological and biochemical changes

At 20 dap, β-galactosidase activity was twice as high in transgenic line 6-2, and 3-fold higher in line 6-10, compared with the wild type (Fig. 6A). However, β-galactosidase activity was virtually the same in transgenics and wild type at later developmental stages (30 dap and Br+3). Exo-galactanase activity was the same in the wild-type and transgenic lines at 20 dap and 30 dap, but at the Br+3 stage, it was reduced in lines 6-2 and 6-10 to 19% and 57% of wild-type levels, respectively (Fig. 6B).

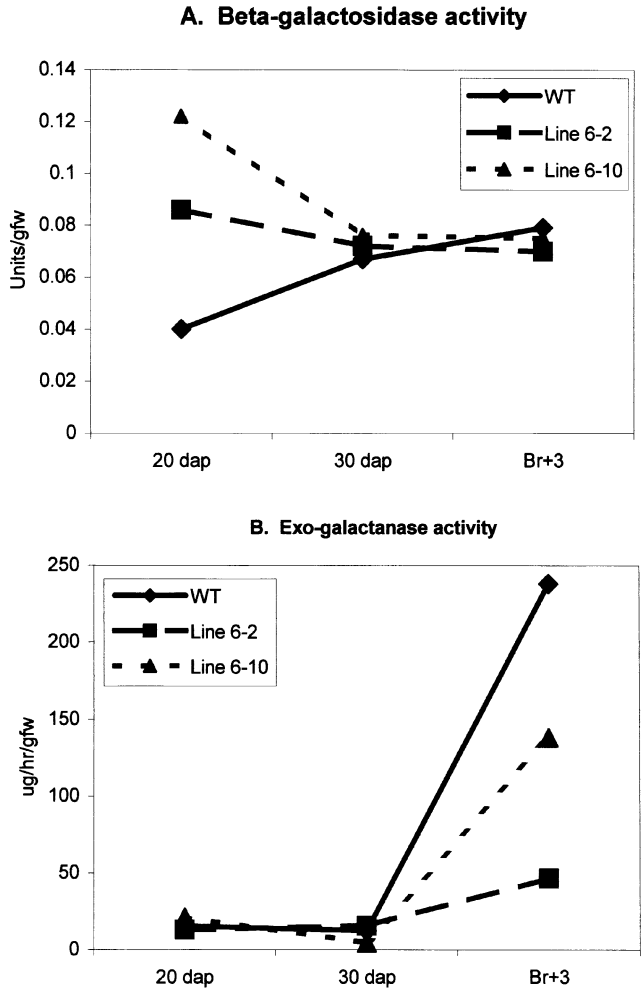

Fig. 6. β-galactosidase and exo-galactanase activity of the wild type and transgenic lines 6-2 and 6-10 at three stages of development (20 and 30 dap and Breaker plus 3 d, Br+3). Enzyme was extracted from control and transgenic fruit tissue at the stages indicated and assayed for total β-galactosidase activity against a PNP-gal substrate (A) and against a lupin galactan for exo-galactanase activity (B). Linear time points for data analysis were determined empirically; assays were performed twice (the average of two assays is shown) and terminated after 2 h (A) and 4 h (B) at 37 °C.

The galactosyl content of the cell wall decreased continually in wild type and transgenic lines throughout development (Fig. 7). However, at 20 dap, transgenic lines 6-2 and 6-10 showed a more marked reduction in cell wall galactosyl content compared with wild type (Fig. 7). Line 6-2 exhibited a 22% decrease, whereas line 6-10 showed a 40% decrease. In all other developmental stages, the galactosyl residue content was the same as in the wild type or azygous fruit.

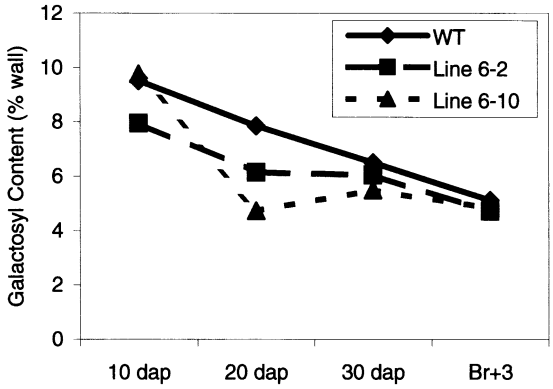

Fig. 7. Galactosyl content in the cell wall of the wild type and transgenic lines at 20 dap, 30 dap and Breaker plus 3 d (Br+3). Galactosyl content is presented as a percentage by weight of the total cell wall.

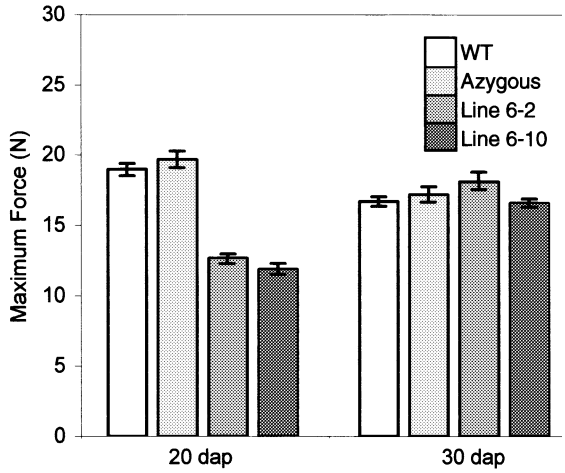

Fig. 8. Whole fruit texture analysis of the wild type and antisense transgenic lines 6-2 and 6-10. Shown are the means of maximum force compression from a 4 mm steel cylinder test (±SE). Two to three measurements of at least 12 fruit per line were taken at 20 dap and 30 dap. Means were tested for statistical significance with the Tukey–Kramer test, with α=0.05.

Lastly, fruit from transgenic lines with suppressed *TBG6* mRNA were significantly softer than control fruit at 20 dap, as shown by the lower maximum force required by a 4 mm cylinder to penetrate 3 mm into the pericarp of the fruit (Fig. 8). Line 6-2 exhibited a 35% reduction in the maximum force, whereas 6-10 showed a 39% reduction. Interestingly, the difference in texture was only found at this stage of development, but not at 30 dap (Fig. 8) or Br+3 (data not shown).

Discussion

The role of the *TBG6* gene product in fruit development was explored by down-regulating its expression. Two antisense lines were found with significantly reduced levels of *TBG6* mRNA. When compared with control fruit at 30 dap, *TBG6* mRNA levels were reduced to 2% and 12% of normal in lines 6-2 and 6-10, respectively. The number of fruit that developed cracks was correlated with decreasing levels of *TBG6* mRNA in the transformed lines; the stronger the suppression, the higher the percentage of fruit exhibiting cracking. Both suppressed lines also exhibited changes in internal fruit morphology (reduced air space in locules) and a doubling of the cuticle thickness in green fruit. A third transgenic line, line 6-3, served as an additional control by expressing *TBG6* antisense mRNA, but not suppressing *TBG6* mRNA or having altered fruit phenotypes.

Several members of the TBG gene family (*TBG1*, *TBG3* and *TBG4*) have been studied by sense co-suppression and antisense suppression. Carey *et al.* (2001) analysed transgenic lines with *TBG1* mRNA levels that were reduced by up to 90%. Even though a yeast-expressed TBG1 gene product was shown to have both β-galactosidase and exo-galactanase activity *in vitro*, total β-galactosidase and exo-galactanase activities, as well as levels of cell wall galactosyl residues and softening, were not affected in the TBG1-suppressed lines (Carey *et al.*, 2001). In another study, down-regulation of *TBG3* mRNA levels resulted in transgenic lines with reduced levels of exo-galactanase activity and increased levels of cell wall galactosyl content (de Silva and Verhoeyen, 1998). Even though the firmness of antisense TBG3 fruit was not significantly affected, the rate of deterioration during long-term storage decreased. Antisense down-regulation of *TBG4* mRNA levels resulted in transgenic plants with fruit up to 40% firmer than controls (Smith *et al.*, 2002). In addition, TBG4 mRNA suppression was correlated with reductions in exo-galactanase activity and levels of free galactose at the mature green stage. These results implied an involvement of *TBG4* in cell wall modification and eventual fruit softening (Smith *et al.*, 2002).

Unfortunately, it was not possible to express *TBG6* using several yeast and *E. coli* expression systems and, therefore, have not confirmed or characterized its activity (data not presented). Nevertheless, of the seven TBGs studied so far, *TBG6* mRNA abundance is highest at the early stages of green fruit development and is, by far, the most abundantly expressed TBG before ripening. The deduced amino acid sequence data predicts the TBG6 product to be 91 kDa and secreted from the cell (Smith and Gross, 2000). More importantly, the TBG6 product is predicted to have β-galactosidase activity because it shares more than 64% sequence identity with other TBGs known to have β-galactosidase and exo-galactanase activity against both native and lupin galactans, such as TBG1, TBG4 and TBG5 (Carey *et al.*, 2001; Smith *et al.*, 1998; Moctezuma *et al.*, 2003) and contains the putative active site-containing consensus sequence pattern G-G-P-[LIVM]-x-Q-x-E-N-E-[FY] belonging to glycosyl hydrolase family

35; where amino acids separated by dashes are conserved, amino acids [LIVM] and [FY] are conserved substitutions, and 'x' is any amino acid (Henrissat, 1998).

For most of the developmental stages tested, β-galactosidase/exo-galactanase activity, cell wall composition, and fruit firmness of control and TBG6-suppressed lines were similar. The most intriguing results obtained in this study were the changes in β-galactosidase activity, cell wall galactosyl content, and fruit texture observed in lines 6-2 and 6-10 at 20 dap. It is interesting that at 20 dap, β-galactosidase activity was higher in lines 6-2 and 6-10 than in the control lines, even though both transgenic lines had very low levels of *TBG6* mRNA, which is the most abundant TBG transcript in the wild type at that particular stage of fruit development. When the mRNA abundance of a target gene is down-regulated by antisense, overall enzyme activity levels may remain normal due to redundancy and/or compensation provided via other members of the gene family. Transcript levels of *TBG4* and *TBG5*, which are expressed in 20 dap fruit and are known to have β-galactosidase activity *in vitro*, were, therefore, examined. However, increased levels of *TBG4* and *TBG5* mRNA were not found in the transgenic lines (Fig. 2). Thus, increased expression of *TBG4* and *TBG5* can not explain why β-galactosidase activity in the transgenic lines was higher than in the wild type in 20 dap fruit. It is possible that the TBG6 gene product activity is not detectable because it does not have activity against the substrates that were tested or activity is lost during the extraction process. It is also not clear why cell wall galactosyl content and fruit firmness are reduced in transgenic fruit only at 20 dap. While the galactosyl levels of the wall were reduced at 20 dap in the antisense lines, free galactose levels remained constant throughout the immature stages of fruit development in all lines (data not shown). However, these observations may have a significant connection with the increased incidence of cracked fruit and thickening of the cuticle in the transgenic lines.

Based on *TBG6* mRNA expression patterns at the early stages of fruit development and the incidence of cracks correlating to decreased levels of *TBG6* mRNA, it is possible that the TBG6 gene product may help to facilitate cell expansion. Previous studies indicate that changes in β-galactosidase activities occur in elongating and rapidly-growing tissues such as epicotyls (Dopico *et al*., 1989). Absence of the TBG6 product may create disturbances in cell wall metabolism during the cell expansion phase of fruit development that are responsible for the morphological changes observed in the antisense lines, such as fruit cracking and the more compact internal fruit anatomy (reduction in open locular space). In addition, it is possible that the TBG6-encoded product is especially critical during the most rapid period of cell elongation and fruit expansion, and its absence may modify fruit texture and/or elasticity, resulting in softer fruit at 20 dap. As fruit continue to develop, the TBG6 product may no longer play a major role in cell expansion, or alternatively, the reduced but not eliminated level of TBG6 product in antisense lines is enough for complete function, and the fruit have normal texture characteristics.

One of the most interesting phenotypes observed was the doubling in cuticle thickness of fruit from *TBG6*-suppressed lines. The cuticle is a film of soluble and polymerized lipids that prevents water loss and provides protection against pathogens and insects (Somerville *et al*., 2000). The outside-facing surface of a typical epidermal cell has a cap of secondary cell wall which is suspected to play a mechanical role in preventing deformation of the epidermis (Kunst and Samuels, 2003). Above the secondary wall, in direct contact with the cuticular lipids and continuous with the pectin-rich middle lamella of the anticlinal walls between adjacent cells, is a pectin-rich layer composed of homogalacturonans and rhamnogalacturonans with cellulose microfibrils branching through it (Kunst and Samuels, 2003). Any modifications to galactan metabolism in these pectin-rich layers could result, for example, in enhanced cuticle deposition to repair the cracks and/or strengthen a weaker structure. Perturbations in fruit growth are known to lead to drastic changes in the composition and depth of the cuticle (E Curry, personal communication). It has also been observed that severe changes in environmental conditions during early apple fruit growth can lead to surface cracking and excess cuticle deposition (E Curry, personal communication). These observations support the idea that the phenotypes observed in *TBG6*-suppressed lines may be due to alterations in cell expansion or orientation during the early stages of fruit development.

The results presented in this study indicate that the presence of a *TBG6* antisense construct may have resulted in decreased levels of *TBG4* and *TBG5* mRNA in fruit, but only at the Br+3 stage. The observed decrease in exo-galactanase activity measured in the transgenic lines at the Br+3 stage of development (Fig. 6), is most likely related to the decrease in *TBG4* and possibly *TBG5* mRNA abundance seen in transgenic line 6-2 at this time point. Smith *et al*. (2002) found that reduced exo-galactanase activity was correlated with reduced *TBG4* mRNA levels in *TBG4* antisense lines, and that the TBG4 product is mostly responsible for the observed exo-galactanase activity in fruit.

It has been suggested that the pectin matrix establishes the 'pore size' in the cell wall, i.e. the relative size of the channels formed by the wall matrix that permit polymeric molecules to diffuse freely through the matrix. This pore size can be established by a combination of the frequency and length of the junction zones, the degree of methyl esterification, and the length of arabinans, galactans, and arabinogalactans attached to rhamnogalacturonan I (RGI) that extend into the pores (Carpita and McCann, 2000). An

increased content of pectic galactan side chains has also been linked to increased mechanical strength of the wall in pea cotyledons (McCartney *et al*., 2000). Despite repeated tries using yeast and *E. coli* systems, it has not been possible to express the TBG6 protein product, in order to characterize enzyme activity, substrate specificity, etc. Even so, the data presented here suggest that the TBG6 product may have an important function in cell wall galactosyl residue metabolism during cell elongation. It is interesting to note that many β-galactosidases have additional specific biosynthetic activities, such as transglycosylation and reverse hydrolysis (Yoon and Ajisaka, 1996). These functions, although not yet identified in plant cell wall metabolism, may have important roles in modifying cell wall structure to accommodate periods of rapid wall modifications which occur, for example, during cell expansion.

In addition, it would be useful to generate transgenic plants that over-express TBG6 during fruit ripening in order to investigate if this leads to reduced cracking during the handling and shipping of post-breaker fruit. It would also be interesting to show how the observed morphological phenotypes can be used for improving commercial tomato varieties. For example, decreased locular space can lead to developing 'meatier' tomatoes, which could have significant impact in the fresh-cut slice industry. Further study in the genetic and molecular origins of increased cuticle thickness, without the deleterious effects of increased cracking, may be helpful in developing fruit with improved resistance to water loss and infection by pathogens. Summing up, the various altered phenotypes observed as a result of *TBG6* down-regulation in tomato fruit are further evidence that β-galactosidases have important functions in the overall growth and development of tomato fruit.

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Note: Use of a product name by the US Department of Agriculture does not imply approval or recommendation of such product to the exclusion of others, which may also be suitable.

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